

CONTINUATION APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: MODIFIED PLANTS

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CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EV321181434US

November 6, 2003
Date of Deposit

MODIFIED PLANTS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of International Application No. PCT/GB00/02953, internationally filed July 31, 2000, which was published in English, and claims priority to Great Britain Application No. 9918061.4, filed July 30, 1999, the disclosures of both of which are incorporated in their entirety by reference hereto.

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for controlling endosperm size and development, and seed viability in plants. The invention also relates to nucleic acid constructs for use in such methods, as well as modified plants *per se*.

Related Art

Yield in crop plants where seed is the harvested product is usually defined as weight of seed harvested per unit area (Duvick, 1992). Consequently, individual seed weight is regarded as a major determinant of yield. Most monocotyledonous plants e.g. maize, wheat, (see Esau, 1965) produce albuminous seeds - that is, at maturity they contain a small embryo and a relatively massive endosperm. Consequently, in monocotyledonous plants, the endosperm represents a significant component of seed yield. Endosperms accumulate and store diverse substances, including starch, proteins, oils and fats.

Therefore, in monocotyledons increasing the size of the endosperm or its ability to accumulate storage products is likely to increase individual seed weight and perhaps total yield.

Endosperms are utilised commercially in diverse ways, either indirectly as part of the whole seed or directly following their purification, or the purification of certain of their constituents. Hence endosperms may represent either a proportion or the entire commercial value of a crop. Examples of indirect usage include fodder maize and whole wheat flour. An example of direct usage of the complete endosperm is in the production of white flour for bread-making. Finally, maize oil represents an example of the utilisation of a constituent of the endosperm, but there are many others.

In contrast to monocotyledons, most dicotyledonous plants, e.g. oil seed rape, soybean, peanut, *Phaseolus vulgaris* (e.g. kidney beans, white beans, black beans), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea), and *Lens culinaris* (lentil) produce exalbuminous seeds - that is, mature seeds lack an endosperm. In such seeds the embryo is

large and generally fills most of the volume of the seed, and accounts for almost the entire weight of the seed. In exalbuminous seeds the endosperm is ephemeral in nature and reaches maturity when the embryo is small and highly immature (usually heart/torpedo stage).

Commonly embryo development depends on the presence of the endosperm, which is generally accepted to act as a source of nutrition for the embryo.

Scott *et al* (1998) showed that the size of the endosperm in terms of the number of endosperm cells at maturity in the dicotyledonous plant *Arabidopsis thaliana*, a close relative of oil seed rape (*Brassica napus*), is positively correlated with the weight of the mature seed. Plants that developed seeds with 80% smaller endosperms (average = 80 nuclei) compared to controls (mean of 2x-2x (diploid plant crosses) and 4x-4x (tetraploid plant crosses) = 400 nuclei) produced seeds that were 46% smaller (in weight terms = 14 μ g) than the controls (mean of 2x-2x and 4x-4x = 30 μ g). In contrast, plants that developed seeds with 160% bigger endosperms (average = 640 nuclei) compared to controls (mean of 2x-2x and 4x-4x = 400 nuclei) produced seeds that were 180% larger (in weight terms = 54 μ g) than the controls (mean of 2x-2x and 4x-4x = 30 μ g). *Arabidopsis* seed in common with most other dicotyledonous seed is composed almost entirely of embryo. Hence the change in seed weight is almost completely due to a change in embryo weight.

Consequently, modifying endosperm size, in terms of the number of cells at maturity, has a dramatic impact on seed weight in seeds that do not contain endosperm at maturity. Without being bound by the following, one reasonable hypothesis is that a larger endosperm accumulates a greater quantity of reserves from the seed parent, perhaps by acting as a stronger "sink". These reserves then provide more resources for utilisation by the growing embryo, resulting in a larger seed. Alternative mechanisms might operate, however.

The seeds of dicotyledons, like those of monocotyledons are utilised in diverse ways. For example, pulses such as soybean, peanut, *Phaseolus vulgaris* (e.g. kidney beans, white beans, black beans), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea),

Lens culinaris (lentil) are important world crops that are used directly for animal and human consumption. Seeds of oil rape, sunflower and linseed are processed to produce oils.

Clearly, despite the differences in the structure of monocot and dicot seeds, particularly with respect to the presence or absence of endosperm in mature seeds, the size of the endosperm is an important factor in determining individual seed weight, and therefore potentially total crop yield in plants where seed is the economic harvest. Indeed, Hannah and Greene (1998) showed that maize seed weight is dependent on the amount of endosperm ADP-glucose pyrophosphorylase, the enzyme responsible for supplying substrate for starch synthesis.

However, there is some evidence that an increase in seed weight is associated with a reduction in seed number in many breeding populations. Consequently, increasing individual seed size may not result in an increase in total yield. While maize breeding programmes have been successful and genetic improvement has played a significant role in increased maize yields, the genetic component to yield has led to only a doubling of this parameter since the 1930s (Duvick, 1992). The increase in maize yields is currently less than 1% per year.

The genetic basis for the resistance to increased seed weight encountered in conventional breeding programmes is not understood. However, Giroux et al (1996) showed that a single gene mutation in the endosperm specific gene *shrunken2* of maize resulted in a seed weight increase of 11-18% without a reduction in seed number. This suggests that yield improvements are possible in a plant with a long history of intensive and successful breeding efforts, and may therefore be generally achievable. Similarly, Roekel et al. (1998) showed that introduction of the *tzs* gene into *Brassica napus* results in a significant increase in seed yield accounted for by increased seed number per silique and increased seed weight.

There is also evidence that seed size (weight) is positively correlated with a number of components of "seed quality" such as percent germination (Schaal, 1980; Alexander and Wulff, 1985; Guberac et al, 1998); time to emergence (Winn, 1985; Wulff, 1986); durability (survival under adverse growing conditions) (Krannitz et al, 1991; Manga and Yadav, 1995);

growth rate (Marshall, 1986) and yield (Guberac et al, 1998). Seed quality is an important factor in the cost of production of commercial seed lots since these must be tested before sale. Consequently, increasing total seed weight, even without increases in total seed yield may have economic benefits through improvements in seed quality.

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We have recently demonstrated (Scott et al., 1998) that hybridising *Arabidopsis* plants of different ploidies has reproducible and dramatic effects on the weight of progeny seed. For example, an interploidy cross between a diploid (2x) seed parent and a tetraploid (4x) pollen parent (2x-4x) results in seed which is 240% larger than 2x-2x seed. Conversely, 4x-2x crosses result in a reduced seed size (60% of 2x-2x). Analysis of endosperm development in these F1 seed reveals a clear correlation between final seed size and the size of the endosperm. In common with most dicots, endosperm is not present in the mature *Arabidopsis* seed but is required to nourish the developing embryo. Therefore, increased endosperm size translates into increased seed size by increasing embryo size, presumably by accumulating and then supplying increased nutrition, or by some other less direct means enabling the embryo to accumulate more resources from the mother.

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In wild type 2x-2x crosses the endosperm is triploid and is formed by the fertilisation of a pair of fused haploid polar nuclei of maternal origin with a haploid sperm of paternal origin.

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Consequently, there is a 2:1 ratio of maternal to paternal genomes in the normal endosperms. An excess of paternal genomes in the endosperm, e.g. as a result of a 2x-4x cross, causes increased endosperm proliferation (hyperplasia). An excess of maternal genomes in the endosperm (4x-2x crosses) has the opposite effect: decreased endosperm proliferation (hypoplasia).

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Scott et al (1998) explain these observations in terms of the genomic imprinting (inactivation) of genes that contribute to endosperm vigour, either positively or negatively. Accordingly, paternal gametes have an overall positive effect on endosperm growth because genes that inhibit endosperm growth or functionality are imprinted, whilst genes that have a positive effect escape imprinting and are active in the endosperm. Adding more paternal genomes into the

endosperm via a tetraploid pollen parent therefore increases the number of stimulatory genes resulting in a larger endosperm. Maternal genomes have the opposite effect. Importantly, imprinting effects have been recorded in a wide range of plant species including maize and brassicas. In mammals, a number of genes that influence foetal growth (typically expressed in the placenta) also exhibit uniparental expression due to imprinting during gametogenesis. Extra doses of these genes also have dramatic effects on embryo size.

Hybridisation is recognised as an important process for producing offspring having a combination of desirable traits from both parents. Hybridisation may be interspecific or intraspecific. Interspecific hybridisation is used for introducing desirable traits such as disease resistance into crop species. However, the ability to make successful sexual crosses is frequently restricted to closely related species because of the existence of a variety of pre-fertilisation and post-fertilisation reproductive barriers (see Stoskopf, Tomes and Christie, 1993). One type of post-fertilisation barrier is associated with poor or disrupted endosperm development (post-fertilisation endosperm development barrier), which results in non-viable seed (see Ehlenfeldt and Ortiz, 1995). Endosperm failure in unsuccessful crosses is due to the operation of a genetically determined system known as endosperm dosage (Haig and Westoby, 1991). Endosperm dosage is a form of genomic imprinting. The removal of the endosperm dosage barrier to sexual interspecific hybridisation would have economic benefits, since non-sexual techniques for hybridisation e.g. somatic hybridisation are costly and difficult.

The endosperm dosage system may also prevent intraspecific hybridisation where the parents are of different genomic constitutions (ploidies) (Haig and Westoby, 1991).

The occurrence of successful intra- and interspecific hybridisation can also be problematic. In particular, hybridisation between genetically modified crop plants and non modified cultivated or wild plants thereby creating hybrids carrying transgenes with the potential for environmental and other damage inherent in this form of "transgene escape", has caused alarm within both the public and the regulatory authorities.

There are various strategies that might be used to prevent transgene escape from crops into the wider environment. Critically, a range or spectrum of methods should be available to meet practical constraints imposed by the requirements of plant breeders and seed producers and the life histories of specific crop species when in the hands of farmers. For example, the complete elimination of flowering is acceptable in vegetable crops and forage grasses during the 'cropping stage', but unless this trait is conditional in some way, the production of seed by the seed producer, or the breeding of new varieties by the plant breeder, is rendered difficult or impossible.

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In crops where the harvest is a fruit or a seed, given that most crop species are self-pollinating, the production of pollen, by at least the majority of flowers, is essential. Most of the major crops fall into this category.

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Cleistogamous plants produce flowers that develop normally but which fail to open.

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Consequently, self pollination occurs, but no pollen escapes from the flower. Whilst this the implementation of this solution would 'only' require modifications to flower design, such an approach might be criticised on the grounds that pollen could escape from damaged flowers.

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The production of viable sexual hybrids occurs within species (intra-specific hybridisation) or between species (inter-specific hybridisation). However, in the case of inter-specific hybridisation, a successful outcome - viable hybrid seed - is usually only possible between closely related species. Two main barriers prevent hybridisation between more widely diverged species - inter-specific incompatibility at the stigma surface or within the style, which prevent fertilisation, and post-fertilisation barriers which cause seed abortion, usually through failures in endosperm development (Brink and Cooper, 1947; Ehlenfeldt and Ortiz, 1995).

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Brink and Cooper (1947) working in *Lycopersicum* were the first to propose that the primary reason for the failure of inter-specific crosses was the same as for intraspecific crosses, namely failure of the endosperm itself. The operation of this type of barrier has been reported in numerous species including the Brassicas (see Haig and Westoby, 1991). These authors and

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others (see Ehlenfeldt and Ortiz, 1995) also proposed that endosperm failure in inter-specific crosses is due to an effective, rather than actual, imbalance in the normal ratio of maternal to paternal genomes in the endosperm. Different species are proposed to have different genomic strengths. Hence a cross between plants of the same ploidy may fail because the relative genomic strengths of their respective genomes result in a lethal effective genomic imbalance within the hybrid endosperm. Likewise a cross between two plants of different ploidies may succeed provided their relative genomic strengths result in a hybrid endosperm with a balanced genomic constitution. The setting of genomic strength is proposed to involve genomic imprinting, although the exact nature of the relationship is not understood.

In summary, the failure of intraspecific (interploidy) crosses and crosses between species may have a common cause – a genomic imbalance within the endosperm mediated by genomic imprinting. Modifying the genomic strength of one or both of a pair of species that normally hybridise may have application in generating a lethal relative endosperm imbalance, thereby creating a post fertilisation barrier between the two species. The same approach may have application in providing a post-fertilisation barrier within a species, for example between genetically-engineered crop varieties and non-engineered varieties. Practically, for transgene containment the genomic strength of the crop could be modified to prevent cross hybridisation with any problematic close relatives. Such a technology would facilitate the exploitation of genetically modified plants, with considerable economic and environmental benefits.

There is currently considerable research effort to develop transgenic technologies (see Koltunow et al., 1995) to introduce apomixis into crop species. In natural apomorphic plant species $2n$ seed is produced without fertilisation of the egg. The genetic constitution of the embryo is therefore identical to that of the seed parent. The economic benefits of introducing an apomixis system into crop species include true breeding F1 hybrids. Currently, F1 hybrid seed is produced annually by hybridising two genetically distinct parents in a labour intensive and costly process. True breeding (apomorphic) F1 hybrids could be propagated for sale without the hybridisation step. The removal of this step would potentially therefore reduce production costs.

An essential aspect of apomixis is that the embryo is derived from a cell with an unreduced ($2n$) number of chromosomes. In natural apomicts this is achieved by modifying meiosis (meiotic reconstitution) such that $2n$ gametes are produced, or deriving the embryo from a somatic cell with the $2n$ number of chromosomes. Irrespective of the origin of the embryo the endosperm is invariably derived via meiosis which is either restitutive or reductional. In the former case the two polar nuclei, which upon fertilisation produce the endosperm, are $2n$ and in the later case n . Given that natural apomicts utilise endosperms generated in this way it is likely to be the case for genetically engineered apomictic crop plants.

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A potential problem in the development of apomictic crop species, given this likely dependency on 'sexual endosperms' (formed by fertilisation), is ensuring the successful development of the endosperm, since the endosperm is required to nourish the embryo or itself represents the principal economic harvest. One barrier to endosperm development is the endosperm dosage system. In species with an endosperm dosage system the ratio of maternal to paternal genomes in the endosperm is 2:1. Deviation from this ratio results in endosperm abortion and seed lethality (Haig and Westoby, 1991). Natural apomicts have adopted a number of strategies to ensure endosperm development. A few species (autonomous apomicts) develop a gynogenetic endosperm (maternal) in the absence of fertilisation of the polar nuclei. The majority however, retain fertilisation of the polar nuclei and maintain a 2:1 genomic ratio by modification of either male meiosis (to produce unreduced gametes) or the fertilisation process e.g. fertilisation involves only 1 polar nucleus. Still other species successfully deviate from the genomic 2:1 ratio.

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For engineered apomixis the most attractive solution for ensuring endosperm development is the provision of autonomous endosperm development. Solutions involving fertilisation of the polar nuclei are likely to complicate the delivery of apomixis, for example by necessitating the introduction of a mechanism to prevent fertilisation of the "egg" or the need to devise ways to produce $2n$ male gametes, or by some other means ensure a 2:1 genomic ratio.

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One approach to developing an autonomous apomict involves the induction and isolation of mutant genes that condition endosperm development in sexual species without fertilisation. Extensive screening efforts in *Arabidopsis* met with limited success having identified several mutant genes that condition only limited endosperm development in the absence of fertilisation
5 (Ohad et al., 1996; Chaudhury et al., 1997; Ohad et al., 1999; Kiyosue et al., 1999; Luo et al., 1999). One potential explanation is that these mutations trigger endosperm development but do not overcome the effects of the endosperm dosage system. Endosperms in the mutants would have a genetic constitution of 2 maternal:0 paternal genomes, which deviates from the normal 2:1 genomic ratio. Significantly, Scott et al, 1998, recently showed that *Arabidopsis* possesses a
10 dosage system capable of causing seed abortion where the ratio of parental genomes in the endosperm deviates significantly from 2:1.

Autonomous apomixis would enable the crop to produce seed without any requirement for pollen. Hence transgene escape through pollen could be prevented by arranging for the crop
15 plant to carry any form of male sterility that stops the production or release of functional pollen.

The interploidy cross effect on seed size, the post-fertilisation endosperm development barrier to interspecific hybridisation and the barrier to autonomous endosperm development are all explicable in terms of genomic imprinting.
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In mammals, a number of genes that influence foetal growth (typically expressed in the placenta) exhibit uniparental expression due to genomic imprinting during gametogenesis. Extra doses of these genes can have dramatic effects on embryo size (Solter, 1998). Genomic imprinting also prevents the development of gynogenetic or androgenetic (two paternal genomes, no maternal genome) embryos (Solter, 1998).
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In mammals, genes selected for imprinting are maintained in an inactive state by DNA methylation. The enzyme responsible is DNA methyltransferase (MET) which is encoded by a single gene. Mice embryos containing an inactive DNA methyltransferase gene die at an early
30 developmental stage and express both parental copies of genes that are normally imprinted (i.e.

uniparentally expressed) (Li et al, 1993). This demonstrates the involvement of DNA methyltransferase in genomic imprinting and a requirement for imprinting in normal development.

5 In plants the imprinting mechanism is unknown. However, plant genomes contain relatively large amounts of the modified nucleotide 5-methylcytosine (Gruenbaum et al, 1981). Despite evidence implicating cytosine methylation in plant epigenetic phenomena, such as cosuppression and inactivation of transposable elements (Napoli et al, 1990; Bender et al, 1995; Brutnell and Dellaporta, 1994, Martienssen et al., 1995; Matzke and Matzke, 1995) the role of cytosine 10 methylation in plant developmental processes and genomic imprinting remains unclear.

To date three different genes have been found that may be imprinted in the maize endosperm: tubulin (Lund et al 1995), a storage protein regulator gene *d2r* (Chaudhuri, and Messing, 1994) and the *r* gene transcription factor that regulates anthocyanin biosynthesis (Kermicle and Alleman, 1990). In each case, the maternally inherited allele is undermethylated, over-expressed or both, whereas the paternally inherited allele is more methylated or has a reduced level of expression.

20 In *Arabidopsis*, *ddm* mutants (decrease in DNA methylation) have been isolated with reduced levels of cytosine methylation in repetitive sequences, although the mutations do not result in any detectable change in DNA methyltransferase activity (Vongs et al., 1993; Kakutani, 1995). After several generations of self pollination, *ddm* mutants exhibit a slight delay (1.7 days) in flowering, altered leaf shape, and an increase in cauline leaf number (Kakutani, et al, 1995). Repeated self pollination of *ddm* mutant plants does however result in the appearance of severe 25 developmental abnormalities (Kakutani et al, 1996).

30 *Arabidopsis* plants expressing a DNA methyltransferase 1 (Met1) antisense (Met1as) gene contain reduced levels of DNA methyltransferase activity and a correspondingly reduced level of general DNA methylation (Finnegan et al, 1995; Ronemus et al., 1996). In contrast to *ddm* mutants, *Arabidopsis* plants expressing a Met1as gene develop various developmental

abnormalities at high frequency and without repeated self-fertilisation, including floral abnormalities (Finnegan et al, 1996). PCT /US97/13358 also reports that *Arabidopsis* plants expressing a Met1as gene alter the rate of development of the plant. The development of the endosperm in *ddm* mutants and plants expressing Met1as has not been reported.

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The present invention is based on the unexpected observation that a decrease of about 90% in the amount of methylated DNA present in a plant genome results in the production of gametes, both male and female, that behave in a manner that is consistent with the removal or attenuation of genomic imprinting. This is exemplified by the following experiments:

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1. Endosperm development in seeds derived from a cross between a wild type 2x plant, as seed parent, and a 2x Met1as plant as pollen parent (2x-2xMet1as), resembles endosperm development in seeds derived from a 4x-2x interploidy cross (Figures 1 and 3). - the endosperm is small/underdeveloped. The resulting seed is smaller in weight terms than seed from control 2x-2x crosses (Table 1). Hence the male gametes from a Met1as plant behave like a female gamete from a wild type plant. This can be explained by proposing the removal or attenuation of imprinting in the male gamete.

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2. Endosperm development in seeds derived from a cross between a 2xMet1as plant, as seed parent, and a wild type 2x plant as pollen parent, strongly resembles endosperm development in seeds derived from a 2x-4x interploidy cross between wild type plants (Figures 1 and 3). - that is, the endosperm is large/overdeveloped. The resulting seed is larger in weight terms than seed from control 2x-2x crosses (Table 1). Hence the female gametes from a 2xMet1as plant behave as a male genome of a normally methylated diploid plant. This can be explained by proposing the removal or attenuation of imprinting in the female gamete.

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3. Reciprocal crosses between 2xMet1as and 4x wild type plants result in seed abortion (Figures 1 and 3); consequently seeds derived from these crosses are shrivelled and do not germinate (Table 1). The behaviour of the endosperm in seed generated in these crosses depends on the direction of the cross. Where the 4x plant is the seed parent the endosperm is

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extremely under-developed and contains very few endosperm nuclei and a very small chalazal endosperm (Figure 1, Table 1). In contrast, where the 4x plant is the pollen parent the endosperm of the resulting seeds is over-developed, and contains many endosperm nuclei and a very well developed chalazal endosperm with many associated chalazal nodules (Figures 1 and 5 3, Table 1). This outcome resembles those obtained in crosses between 2x and 6x wild type plants which routinely fail to produce viable seed (Figure 3) and display very under- (6x-2x) or over-developed (2x-6x) endosperm depending on the direction of the cross. These crosses represent examples of lethal parental genomic excesses within the endosperm that result from 10 the large disparity between the ploidy level of the respective parents. The similarity between 10 outcomes and the behaviour of the endosperm in 2xMet1as -4x and 2x-6x reciprocal crosses can be explained by proposing that male and female gametes derived from 2xMet1as plants behave, in part, like gametes of the opposite sex with respect to genomic imprinting. This again strongly suggests that DNA hypomethylation caused by the Met1as gene removes or strongly attenuates genomic imprinting.

15 4. The behaviour of plants homozygous for the *ddm* mutation in reciprocal crosses with 2x and 4x wild type plants is very similar to that of plants homozygous for the Met1as gene (see Figure 2 and Table 1). This strongly suggests that the basis of the interploidy cross effect is associated 20 with general DNA hypomethylation.

20 Thus, in a first aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, 25 with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of the resultant plant and one or more sequences whose expression or transcription products(s) is/are capable of modulating genomic imprinting.

30 As will be described herein, modulation of imprinting of plant gamete DNA can be used alter endosperm development. The effects can be applied to male or female gametes of the transformed plant. Thus, in a second aspect, the present invention provides a method for the

production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing gynoecium, especially the cell lineage that gives rise to the female germ line (megasporocyte tissue), within the ovule of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of modulating genomic imprinting.

In a third aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing stamen, especially the cell lineage that gives rise to the male germ line (microsporocyte tissue) of the resultant plant and one or more sequences whose expression or transcription product(s) is/are capable of modulating genomic imprinting.

There are a number of proteins known or suspected to be involved in the process of genomic imprinting. Altering the rate of expression of those genes in the germ line of either sex can also be used to alter the development of the endosperm in a parent-specific manner.

In the African claw toad *Xenopus laevis*, the product of the methyl-cytosine binding protein 2 (MeCP2) has been shown to specifically bind to methylated cytosines (Kass et al., 1997; Jones et al., 1998). This protein, of which conserved homologs in mammals also exist, forms a complex at the C-met locus with several other proteins. Amongst these are the transcription-repression mSin3 proteins (Nan et al., 1998; Laherty et al., 1997) and a number of histone deacetylases (HDAC). The activity of the latter genes is presumed to be an important step in the process of anchoring histones to the DNA and hence the formation of heterochromatin and the silencing of genes (reviewed in Razin, 1998 and Pazin and Kadonaga, 1997). The MeCP2-protein may thus constitute the first step in the gene silencing process by guiding the heterochromatin-forming machinery to C-met loci. Interestingly, in contrast with this the

protein has also been found to have a de-methylating function in that it removes methyl-groups from cytosine residues (Bhattacharya et al., 1999).

If the homologs of proteins in the C-met binding complex in plants are likewise involved in uniparental gene silencing (imprinting) then inactivation of these genes in the maternal or paternal germ lines would be predicted to mimic the uniparental inactivation of the genes responsible for methylation. In addition, there could be a cumulative effect if more than one gene is inactivated. If for instance inactivation of the MET1 gene by antisense transcription or ds-RNA in one of either germ lines is not complete, then introduction of an additional vector causing inactivation of one of the other components of the imprinting machinery will enhance the effect.

In a preferred aspect, the present invention provides a method for the production of modified endosperm based on targeting the germ line or gametes with transgenes which alter the capacity of genes to form, maintain or express imprints. This can be achieved in a number of ways. Firstly, by incorporation of one or more sequences encoding proteins associated with the application or maintenance of genetic imprints. Specifically, such sequences may encode a histone deacetylase, methyl cytosine binding protein or Sin 3 proteins, for example, m Sin 3.

Alternatively, the transgene may incorporate sequences including the FIE gene or the FIS gene, for example fis1, fis2 or fis3.

Imprinted genes may also contain, or be located close to, signals within the DNA sequence (a particular nucleotide sequence motif) that mark them out for imprinting during gamete production. Such a motif may, in addition to expressed proteins associated with the formation and/or maintenance of genomic imprints, be involved in the formation of an "imprinting complex". It is contemplated that removing or inactivating the DNA motif, or restricting the availability of the associated proteins, in the imprinting complex may provide a means for preventing or attenuating the application of imprints, thereby allowing the expression of genes which may otherwise be silenced in the endosperm.

The present invention further provides methods for removing or attenuating genomic imprinting, based on targeting the germ line or gametes with transgenes which alter the methylation pattern of genes, or their capacity to form or maintain imprints, within the developing endosperm. Thus, in a fourth aspect, the present invention provides a method for the production of modified endosperm, which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of the resultant plant, and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

The restriction of imprint removal or attenuation to one or other sex of gamete is desirable for 3 reasons:

- 15 1. To provide for removal of imprinting in a single sex of gamete within an individual plant. This will produce the asymmetry of imprinting that is required to mimic the interploidy cross effect in a self-fertilising plant.
- 20 2. To prevent developmental abnormalities that are associated with generalised hypomethylation, such as occurs with the CaMV35S driven Met1 antisense gene.
- 25 3. To prevent the attenuation of the interploidy cross effect due to the expression of the hypomethylation gene (Met1as) within the endosperm. Crosses between two 2xMet1as plants result in seed with a slightly increased number of endosperm nuclei and normal seed weight (Table 1), which is most easily explained by proposing that the combination of hypomethylated gametes of both sexes allows normal endosperm development

The important property of the nucleic acid molecule used in the transformation step is that DNA of cells that contribute to one sex of germ line is subject to alteration of the pattern of DNA methylation through the activity of the transgenes. The germ-line is the tissue within the

reproductive organs that produces the gametes. In the anthers (stamen) this is the microsporogenous cell tissue and in the pistil (gynoecium) the megasporocyte tissue.

Since the timing of the application of the genomic imprints is currently not known the activity
5 of the regulatory sequences, e.g. promoters (or fragments of promoters) promoters should be as broad as possible whilst remaining consistent with the principles discussed herein.

As will be described herein, alteration of the methylation of plant gamete DNA can be used to modify endosperm development. Thus, in a fifth aspect, the present invention provides a
10 method for the production of modified endosperm, which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing gynoecium, especially the cell lineage that gives rise to or comprises the female germ line (megasporocyte tissue), within the ovule of the resultant plant, and one or more sequences encoding one or more
15 proteins which cause methylation or demethylation of nucleic acid.

In this aspect of the invention, the resultant endosperm is larger, and the seed produced is heavier. Herein, suitable promoters include promoter fragments from the *Arabidopsis* AGL5 gene (Sessions et al., 1998), the *Petunia* FBP7 and FBP11 genes (Angenent et al., 1995;
20 Colombo et al., 1995), *Arabidopsis* *BEL1* gene (Reiser et al., 1995), *Arabidopsis* *MEDEA* (*FIS1*) gene (Grossniklaus et al., 1998; Kiyosue et al., 1999), *Arabidopsis* *FIS 2* (Kiyosue et al., 1999), *FIE* (*FIS 3*) (Ohad et al., 1999; Kiyosue et al., 1999), orthologs/homologues of these genes from other species. Other promoters that drive expression that is restricted to cells within the female reproductive organs that contribute to the female germ line would also be
25 suitable. Especially suitable are promoters from gynoecium-specific genes that are first expressed during early gynoecium development, preferably before the differentiation of individual ovules, and which maintain their expression until ovule differentiation is complete (contain egg cell and binucleate central cell).

As used herein, the term "homologues" of the genes is defined to include nucleic acid sequences comprising the identical sequence to the gene or a sequence which is 40% or more identical, preferably though 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% to the sequence of the gene at the nucleic acid residue level, using the default parameters of the GAP computer program, version 6.0 described by Devereaux *et al.*, 1984 and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilises the alignment method of Needleman and Wunsch 1970 as revised by Smith and Waterman 1981.

5

In a sixth aspect, the present invention provides a method for the production of modified endosperm which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable of directing expression within the developing stamen, especially the cell lineage that gives rise to or comprises the male germ line (microsporocyte tissue) of the resultant plant and one or more sequences encoding one or more proteins which cause methylation or demethylation of nucleic acid.

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15

In this aspect of the invention, the resultant endosperm is smaller, and hence the seed is lighter. Herein, suitable promoters include promoter fragments derived from the *Arabidopsis* genes *APETALA3* (Jack *et al.*, 1992; Irish and Yamamoto, 1995), the *Arabidopsis PISTILLATA* gene (Goto and Meyerowitz, 1994), the *Arabidopsis E2* (Foster *et al.*, 1992), the *Arabidopsis APG* (Roberts *et al.*, 1993), homologues/orthologs of these genes from other species. Other promoters that drive expression that is restricted to cells within the male reproductive organs that contribute to the male germ line would also be suitable. Especially suitable are promoters from stamen-specific genes that are first expressed during early stamen development, preferably before the differentiation of individual microsporocytes, and which maintain their expression until stamen differentiation is complete.

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25

Herein, promoters that drive gene expression in cells of the germ line or in cells that represent the direct progenitors of the germ line within either the stamen or pistil and which, when in

conjunction with the Met1as gene, produce hypomethylated gametes are referred to as 'germ line' promoters.

Thus, as will be appreciated by the skilled person, the present invention allows for the modification of the endosperm such that it is either increased or decreased in size. In addition, the development of the endosperm can be altered such that the modified plants can be used in carrying out intraspecific hybridisation, erecting artificial barriers to intra- and interspecific hybridisation to prevent "transgene escape", or in engineering apomixis.

In one specific embodiment, the degree of methylation is increased. This can readily be achieved by incorporating one or more sequences encoding one or more methylating enzymes into the transgene.

Examples of suitable methylating enzymes include:

- i)Methylase 1 (acc. nr.. C10692);
- ii)Methylase 1-like gene (acc. nr. Z97335);
- iii)Methylase 2 (acc. Nr AL021711); and
- iv)Chromomethylase (acc. Nr. U53501);

all from *Arabidopsis*.

In another specific embodiment, the degree of methylation is decreased. This can be achieved in a number of ways. Firstly, by incorporation of one or more sequences encoding one or more demethylating enzymes, such as de-methylase (= MeCP2-homologue; see below) (acc. nr. AL021635) into the transgene. Alternatively, the transgene can incorporate sequences which cause down regulation of methylating enzymes already present in the plant. For instance, one can use antisense sequences, e.g. the Met1as "gene". In addition, it has been found that incorporation of whole or partial copies of an already present gene can result in suppression of gene expression. Thus, the transgene can incorporate additional copies, or partial copies, of

genes encoding methylating enzymes already present in the plant. In another alternative, the transgene can incorporate a sequence encoding a ribozyme.

- 5 With respect to the sequence, or sequences capable of altering the degree of methylation, sequences encoding methylating or demethylating enzymes can be used. Examples of the latter include:
- 10 i) Methylase 1-like gene (acc. nr. Z97335);
ii) Methylase 2 (acc. nr. AL021711);
iii) Chromomethylase (acc. nr. U53501);
iv) de-methylase (= MeCP2-homologue; see below)(acc. nr. AL021635);

In *Arabidopsis*, possible homologs of the following genes have been found:

- 15 MeCP2 (acc nr. AL021635)
HDAC1/2 (acc. nr. AF014824 & AL035538)
mSIN3 (acc. nr. AC007067_5 & AC002396)
p300: a histone acetylation-gene (acc. nr. AC002986.1 & AC002130.1)
- 20 In a seventh aspect, the present invention provides an isolated or recombinant nucleic acid molecule, eg a DNA molecule, which comprises one or more regulatory sequences capable of directing expression in the male or female germ line and/or gametes of a plant and one or more sequences capable of altering the degree of methylation of nucleic acid.
- 25 In a preferred embodiment of the seventh aspect, the degree of nucleic acid methylation is decreased. An eight aspect of the present invention provides the use of a transgene in which the degree of nucleic acid methylation is decreased, as a post-fertilisation barrier to hybridisation, for example, interspecific or intraspecific hybridisation between plants.

The expression "barrier" is defined to include all forms of reproductive barrier which are associated with poor or disrupted endosperm development. Specifically, the term barrier refers to a post-fertilisation endosperm development barrier, which results in non-viable seed.

5

The transgene provides a barrier to hybridisation by modifying the genomic strength of one or both a pair that normally hybridise thereby causing an effective genomic imbalance leading to failed or disrupted endosperm development. The genomic strength may be modified by removing or attenuating genomic imprinting through DNA hypomethylation. The advantage of preventing hybridisation between plants of the same species (interspecific hybridisation) is discussed earlier in the application in the context of preventing transgene escape.

10

In a ninth aspect, the present invention provides the use of a transgene in which the degree of nucleic acid methylation is decreased, in overcoming a post-fertilisation barrier to hybridisation. In this context, the barrier to hybridisation between plants of the same species (interspecific hybridisation) arises through endosperm dosage which leads to failed endosperm development. The removal or attenuation of genomic imprinting through DNA hypomethylation, may remove the endosperm dosage barrier to interspecific hybridisation. The removal of the endosperm dosage barrier to several interspecific hybridisation would have economic benefits as discussed previously in the application.

20

The nucleic acid of the seventh aspect of the invention will normally be employed in the form of a vector and such vectors form a further aspect of the invention.

25

The vector may be for example a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected or transformed and to enable the selection of cells harbouring vectors incorporating heterologous DNA. Examples of such a marker gene include antibiotic resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the gametes, thus allowing selection of cells or

30

tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

5 Cloning vectors may be introduced into E. coli or another suitable host which facilitate their manipulation. DNA in accordance with the invention will be introduced into plant cells by any suitable means. Thus, according to yet a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention.

10 DNA may be transformed into plant cells using a disarmed Ti-plasmid vector and carried by agrobacterium by procedures known in the art, for example as described in EP-A0117618 and EP-A-0270822. Alternatively the foreign DNA could be introduced directly into plant cells using a particle gun. This method may be preferred for example when the recipient plant is a monocot.

15 A whole plant can be regenerated from a single transformed plant cell, thus in a further aspect the present invention provides transgenic plants (or parts of them such as propagating material) including DNA in accordance with the invention. The regeneration can proceed by known methods. When the transformed plant flowers it can be seen to be male sterile by the inability to produce viable pollen. Where pollen is produced it can be confirmed to be non-viable by the 20 inability to effect seed set on a recipient plant.

25 The present invention also provides transgenic plants and the sexual and/or asexual progeny thereof which have been transformed with a recombinant DNA sequence according to the invention. The regeneration of the plant can proceed by any known convenient method from suitable propagating material.

30 A further aspect of the present invention provides a method for manipulating genomic imprinting in a plant, which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising one or more regulatory sequences capable

of directing expression in the male or female germ line and/or gametes of the resultant plant, and one or more sequences whose expression or transcription product(s) is/are capable of altering the degree of methylation of nucleic acid.

5

Preferred features for each aspect of the invention are as for each other aspect *mutatis mutandis*.

10

The present invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention. The examples are accompanied by the following figures:-

15

FIGURE 1 – Embryo and endosperm development following crosses with *met1*-antisense expressing plants as a parent. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo; column 2, chalazal endosperm; column 3, peripheral endosperm. Note a paternal excess phenotype (over developed chalazal endosperm, highly proliferated peripheral endosperm) in crosses with a demethylated plant as the mother (row 1, 2) and a maternal excess phenotype (small or absent chalazal endosperm and a poorly developed peripheral endosperm) in crosses with a demethylated plant as the father (row 4,5). See text for full details.

20

FIGURE 2 – Embryo and endosperm development following crosses with *ddm1*-mutant plants as a parent. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo; column 2, chalazal endosperm; column 3, peripheral endosperm. See text for full details.

25

FIGURE 3 – Embryo and endosperm development following interploidy crosses and balanced crosses. Confocal micrographs of Feulgen-stained seeds 4-6 days after pollination. Column 1, embryo + peripheral endosperm; column 2, chalazal endosperm. For the 2x-4x and 2x-6x crosses (row 6, 7) the peripheral endosperm is shown as an inset. See text for full details.

30

FIGURE 4 - Schematic diagram showing the method of construction of pAGL5-bin.

FIGURE 5 - Schematic diagram showing the method of construction of pAP3-bin.

5 **FIGURE 6 - Schematic diagram showing the method of construction of pAGL5-asMET1**

FIGURE 7- Schematic diagram showing the method of construction of pAP3-asMET1

10 **FIGURE 8 - Seed production following inter-specific crosses between *Arabidopsis thaliana* and *Arabidopsis lyrata*. Light micrographs of seeds taken from mature seed pods. A, 4x *A. thaliana*X *A. lyrata*; note seeds are shrivelled (see Table 3 for germination data). B, 4x *A.thaliana* Met1a/s X *A. lyrata* (4x *A.thaliana* Met1a/s = hypomethylated tetraploid line expressing Met1 a/s gene); note that seeds are plump (see Table 3 for germination data). See text for full details.**

15 **FIGURE 9 - Seed production following inter-specific crosses between *Arabidopsis thaliana* and *Cardaminopsis arenosa*. Light micrographs of seeds taken from mature pods. A, 4x *A.thaliana*X *C. arenosa*; note seeds are plump (see Table 3 for germination data). B, 4x *A.thaliana* Met1a/s X *C.arenosa*; note seeds are shrivelled (see Table 3 for germination data).**

20 **Figure 10 - Seeds from a *fie-1/FIE* X *FIE/FIE* cross. (A) Light micrograph showing the two classes of seeds, plump (pl) and shrivelled (sh). (Bar = 5 mm). (B-G) Confocal micrographs of normal (B-D) and aborting (E-G) seeds at 8 DAP, centred on micropylar (B, E), central (C, F), and chalazal (D, G) regions of the embryo sac. The endosperm in (E-G) is overgrown and has not cellularized. Bar = 50 μ m.**

25 **FIGURE 10 - Seeds from a *fie-1/FIE* X *FIE/FIE* cross. (A) Light micrograph showing the two classes of seeds, plump (pl) and shrivelled (sh). (Bar = 5 micrometers). (B-G) Confocal micrographs of normal (B-D) and aborting (E-G) seeds at 8 DAP, centred on micropylar (B, E), central (C, F), and chalazal (D,G) regions of the embryo sac. The endosperm in (E-G) is overgrown and has not cellularized. Bar = 50 micrometers.**

FIGURE 11 - Seeds from a [*fie-1/FIE* X *FIE/FIE*; *METI* a/s/*METI* a/s] cross. (A) Light micrograph showing the two classes of seeds. All seeds are plump, indicating that a pollen parent hypomethylated by the *METI* a/s transgene can rescue *fie-1* mutant seeds. Bar = 5 mm.

(B) Identification of the *fie-1* and *FIE* alleles by PCR and restriction enzyme analysis. The wild type *FIE* allele produces four bands (lane 1, WT) while *fie-1/FIE* heterozygotes (lane 2, Het) have an extra band. All large seeds scored had the heterozygous pattern (lane 3) while all small seeds were wild type (lane 4). (C–H) Confocal micrographs of seeds at 8 DAP. The seed in (C–E) has a similar phenotype to seeds from interploid crosses generating maternal genomic excess, while (F–H) shows characteristics of paternal excess (see text, and Scott et al., 1998). Bar = 50 µm.

FIGURE 12 - Autonomous endosperm development in unfertilised seeds of *Arabidopsis thaliana*.

Confocal micrographs of fertilization-independent seeds produced by emasculated *fie-1/FIE* heterozygotes with normal and reduced methylation. (A–C) Seed-like structure from a plant with normal methylation. (A) Optical section showing peripheral endosperm but no well differentiated chalazal endosperm. Bar = 50 µm. (B) Clustered endosperm nuclei at periphery. (PE, peripheral endosperm.) Bar = 50 µm. (C) Endosperm at micropylar (MP) and chalazal (CHP) poles. (D–G) Seed-like structures from *fie-1/FIE*; *METI* a/s plants. (D, E) Type 1 seed-like structures at 7 (D) and 10 (E) days after emasculation (DAE). In these the endosperm cellularizes and fills the interior of the embryo sac. (F, G) Type 2 seed-like structures at 7 (F) and 10 (G) DAE. These produce micropylar and chalazal in addition to peripheral endosperm.

Example 1 The use of gametes from hypomethylated plants (*Met1as* and *ddm*) mimics the interploidy cross effect (alters number of endosperm nuclei formed and consequently the weight of mature seed).

Reciprocal interploidy (different ploidy) crosses between diploid (2x), and tetraploid (4x) (Scott, *et al* 1998) or hexaploid (6x) (Scott, *et al* 1998) *Arabidopsis* plants result in changes to both the size of the endosperm, in terms of the number of endosperm nuclei and volume of the chalazal endosperm, and to the dry weight of mature seeds (see Table 1) and the viability of the seed (Table 1). This is the interploidy cross effect.

Crosses involving Met1as plants

Intraploidy (same ploidy) crosses between 2x Met1as plants and 2x wild type plants mimic this effect (see Table 1 and Figures 1 and 3). A cross between a 2x Met1as plant as seed parent and a 2x wild type plant as pollen parent produces seeds with an average of 450 endosperm nuclei (an increase of 130% over 2xmet-2xmet cross), a relative increase in chalazal endosperm volume of 75% compared to 2xmet-2xmet seed, and a mature dry weight of 20 µg (an increase of 33% compared to seed from 2xmet-2xmet cross) (see Table 1).

A cross between a 2x wild type plant as seed parent and a 2x Met1as plant as pollen parent produces seeds with an average of 200 endosperm nuclei (a reduction of 43% over 2xmet-2xmet cross), a relative decrease in chalazal endosperm volume of 50% compared to 2xmet-2xmet seed, and a mature dry weight of 10 µg (a decrease of 30% over a wild type 2xmet-2xmet cross) (see Table 1).

Table 1. Outcomes of control crosses and crosses involving Met1 antisense and *ddm* mutant plants

Cross	Interploidy cross phenotype ¹	Viability of hybrid seed (%) ²	Maximum number of peripheral endosperm nuclei ³	Relative volume of chalazal endosperm ⁴	Relative change to cellularisation time (days) ⁵	Seed weight (µg) ⁶
2x-2x	NA	95-100	400	1	0	22
4x-4x	NA	95-100	400	2.5	0	36
6x-6x	NA	95-100	300	3.5	0	44
2x-4x	PE	95-100	640	2	+1	54

4x-2x	ME	95-100	80	0.6	-1	14
2x-6x	PE	0 ⁷	400	6.8	absent	6
6x-2x	ME	0 ⁷	50	0.2	-1.5	4
2xmet-2xm _c	PE	95-100	350	1	0	15
2xmet-2xm _c		(90) ⁸	(598) ⁸			(13.6) ⁸
2x-2xmet	ME	95-100	200	0.5	-0.5	10
2xmet-2xm _c		(93) ⁸	(227) ⁸			(9.5) ⁸
2xmet-2x	PE	95-100	450	1.75	+0.5	20
2xmet-2xm _c		(97) ⁸	(1,365) ⁸			(32.5) ⁸
2xddm-2xddm	PE	95-100	350	1.25	0	19
2x-2xddm	ME	95-100	250	0.5	-0.5	12
2xddm-2x	PE	95-100	400	2	+0.5	21
4x-2xmet	ME	0 ⁷	100	0.3	-1.5	3
2xmet-4x	PE	0 ⁷	740	4.4	> +3	15
4x-2xddm	ME	0 ⁷	150	0.3	-1.5	5
2xddm-4x	PE	0 ⁷	680	3.5	> +3	5

NA, not applicable; PE, paternal excess; ME, maternal excess.

1, either paternal (PE) or maternal (ME) excess as defined in Scott et al., 1998. 2, determined by germination on soil. 3, counts done as described in Scott et al., 1998. 4, calculated relative to amount in 2x-2x control cross at heart stage (approx. 5 DAP). 5, expressed relative to 2x-2x control cross (usually 5 DAP). 6, measured as described in Scott et al., 1998. 7, seeds shrivelled. 8, this experiment was performed later subsequent to the experiment that yielded the non-bracketed data and use improved growing technique for the met1a/s plants. This resulted in more vigorous plant which presumably accounts for the observed changes in seed weight. Note however that the changes are qualitatively the same as the original experiment ie. 2x-2xmet are small than 2xmet-2xmet and 2xmet-2x are larger.

15 The presence and (possible) activity of the Met1a/s gene within the endosperm potentially complicates the interpretation of the data produced in out crosses involving homozygous Met1a/s plants. In such crosses the endosperm (and embryo) inherit a single copy of the Met1as, either from the seed or pollen parent. If the Met1as is active within the endosperm it may,

1. disrupt endosperm development since Met1as plants show various vegetative and floral abnormalities associated with the mis-expression of certain genes that regulate development (Finnegan, 1996). However, the presence of the Met1as gene does not appear to have this effect since the endosperms of seeds derived from self pollinated Met1as plants appear developmentally normal except for a degree of paternal excess (Figure 1).
- 5
2. attenuate the magnitude of the interploidy cross effect, by demethylating and thereby erasing imprints from the genome contributed by the normally methylated parent. The imprints must be maintained and propagated in the endosperm if the interploidy cross effect is to be
- 10
- mimicked. The removal of imprints via the action of the Met1as gene could reactivate imprinted loci such that the endosperm genomes behave as if derived from same ploidy parents.

To demonstrate that the interploidy cross effects described above are due to the effect of the Met1as gene on the imprinting of gametes rather than any effect within the endosperm we present data from crosses involving plants hemizygous (that is carrying a single copy) of the Met1as gene. Such plants show patterns of general DNA demethylation similar to homozygotes. Hence gametes derived from these plants are generated in a hypomethylating environment, but because the plants are hemizygous only 50% of these gametes contain the Met1as gene. This enables gametes to be produced in a demethylating environment which then do not subsequently contribute a Met1as into the endosperm when used in crosses. This allows the effect of removing imprints within the gametes to be evaluated in endosperms that do not contain the Met1as gene.

The results of reciprocal crosses involving hemizygotes and 4x wild type plants are shown in Table 2. Both crosses result in a 1:1 ratio of plump, viable : shrivelled, inviable seed. The shrivelled seeds are assumed to result from lethal parental excess caused by the union of a hypomethylated gamete from the hemizygote and a 2x gamete from the 4x parent. Conversely, the plump seeds are assumed to result from normally methylated gamete from the hemizygote and a 2x gamete from the 4x parent. Met1as plants appear therefore to produce both normally methylated and hypomethylated gametes. The plump seeds produce plants which segregate 1:1

for the Met1as gene. Presumably, the shrivelled seeds also segregate 1:1 for the Met1as gene. This data therefore demonstrates that the presence of the transgene in the endosperm is not responsible for the lethality phenotype associated with 2xMet1as-4x reciprocal crosses. If this were the case, seeds containing the Met1as gene would not be recovered among the plump, 5 viable seed class.

Crosses involving ddm mutant plants

Table 1 shows that crosses between wild type diploid and wild type tetraploid plants and plants 10 homozygous for the ddm mutation have very similar outcomes to crosses involving plants containing the Met1as gene. The common feature of the ddm mutation and the action of the Met1as gene is that plants containing these genes have highly hypomethylated DNA. This shows that the interploidy cross effect produced in crosses involving gametes derived from ddm and Met1as plants is related to DNA hypomethylation.

The hemizygote data (Table 2) further suggests that the phenomenon involves hypomethylation 15 of the gametes, presumably through the removal of genomic imprints.

Table 2. Outcomes of reciprocal crosses between *Arabidopsis* plants hemizygous for the 20 Met1as gene and wild type 4x plants.

	Mature Seed phenotypes (%) ¹		Seed viability (%) ²		Proportion viable seeds carrying Met1as gene (%) ³	Seed weight (μg) ⁴	
	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled seeds		Plump seeds	Shrivelled seeds
4x-2xmetHET	50	50	95-100	0	50	11	2
2xmetHET-4x	50	50	95-100	0	50	23	8

Abbreviations: 2x, wild type diploid plant; 4x, wild type tetraploid plant; 2xmetHET, plant 25 hemizygous for the Met1as gene

1, scored by eye. **2**, determined by germination on soil of seed from mature pods. **3**, determined by PCR analysis on plants germinated from plump seeds. **4**, measured as described in Scott et al., 1998.

5

Example 2 Construction of expression cassettes that restrict gene expression to either the gynoecium or the stamen.

10 Example 1 demonstrates that uniparental demethylation can be used to control seed size. However, the increase in seed weight in the cross 2xmet1a/s-2x is smaller than for the corresponding interploid cross (2x-4x). This may be due to the reduced fitness of the 35SMet1as female lines since demethylation is approximately constitutive. In order to reduce and eliminate this effect and to allow seed size changes to be obtained in a single plant it is
15 necessary to restrict demethylation as much as possible to the germ line or gametes.

a. Designing a general female-germ line specific expression vector

An expression vector based on the female-specific AGL5 promoter (Sessions et al (1998)) is constructed as described below. The nos polyA signal sequence is excised from pCaMVNEO (Fromm et al (1986)) as a BamHI, HindIII fragment and cloned between the BamHI and HindIII sites of pBin19 (Bevan 1994) forming pNosterm-bin. A 2.2kb AGL5 promoter is PCR'd from Arabidopsis genomic DNA using the primers AGL5F and AGL5R which introduce an EcoRI and a KpnI site at the ends of the AGL5 PCR fragment.

5' CCGAATTCTCAAGCAAAAGAATCTTGTGGGAG 3' AGL5F

EcoRI

5' CGGTACCTATAAGCCCTAGCTGAAGTATAAACAC 3' AGL5R

KpnI

The AGL5 PCR fragment is cloned as an EcoRI, KpnI fragment between the EcoRI and KpnI sites of pNosterm-bin forming pAGL5-bin (figure 4).

30

b. Designing a general male-germ line specific expression vector

An expression vector based on the male-specific AP3 promoter (Irish and Yamamoto (1995)) is constructed as described below. A 1.7 kb AP3 promoter is PCR'd from *Arabidopsis* genomic DNA using the primers AP3F and AP3R which introduce an EcoRI and a KpnI site at the ends of the AP3 PCR fragment.

5' CGGAATTCAAGCTTCTTAAGAATTATAGTAGCACTTG 3' AP3F

EcoRI

5' GGGTACCTTCTCTTTGTTAATCTTTTGTGAAGAG 3' AP3R

KpnI

The AP3 PCR fragment is cloned as an EcoRI, KpnI fragment between the EcoRI and KpnI sites of pNosterm-bin forming pAP3-bin (figure 5).

Example 3: Construction of chimaeric gene fusions between the female (Example 2a) and male (Example 2b) germ-line specific cassettes and the Met1 antisense gene.

15 Expression of the MET1 gene can be reduced in the female or male germ lines by employing techniques known in the art. For example MET1 down-regulation can be achieved by expressing antisense MET1 or antisense MET1 fragments or sense MET1 or partial sense MET1 or ribozymes directed against MET1 or combinations of the preceding, from promoters expressed in the required germ-line. Below is an example of an antisense MET1 approach.

20

a) The construction of a female germ-line specific Metlas gene

The MET1 cDNA is 4.7 kb long and is isolated by RT-PCR from *Arabidopsis* cDNA using the primers MET1F and MET1R.

25

5' ACTCGAGATTGAAAATGGTGGAAAATGGGGC 3' MET1F

XhoI

5' ACCCGGGTGGTATCTAGGGTTGGTGTGAGGAG 3' MET1R

SmaI

The resulting MET1 PCR fragment is then cloned as a SmaI, XhoI fragment between the SmaI and SalI sites of pAGL5-bin forming pAGL5-asMET1 (figure 6).

5 b) The construction of a male germ-line specific Met1as gene

The MET1 PCR fragment is cloned as a SmaI, XhoI fragment between the SmaI and SalI sites of pAP3-bin forming pAP3-asMET1 (figure 7).

10 Example 4: Introduction of female and male germ-line specific demethylating genes into transgenic plants

Chimaeric genes were introduced via *Agrobacterium*-mediated transformation into wild type diploid *Arabidopsis* using well known techniques.

15 a) pAGL5Met1as

Transgenic *Arabidopsis* plants containing the pAGL5Met1as gene were vegetatively normal and produced flowers with the normal complement of floral organs.

Arabidopsis containing pAGL5Met1as were pollinated with pollen from wild-type diploid plants or allowed to self pollinate. Endosperm development in the resulting seeds was monitored by confocal microscopy (Scott et al., 1998) and seed weights were measured at maturity. In both cases, endosperms showed a paternal excess phenotype (average maximum endosperm size = 800 nuclei, delayed cellularisation (+1-2 days relative to 2x-2x crosses wild type) and chalazal endosperm hyperplasia) similar to that obtained in 2x-4x crosses between wild type plants (Table 1).

25 The mean weight of mature seed collected from pAGL5Met1as plants was 40 µg, compared with a mean of 22µg for 2x-2x seed. This represents an increase in seed weight compared to the mean of the 2x-2x .

The germination frequency was comparable to that of seed from 2x-2x wild type crosses - 95-100%.

The outcomes of the crosses were variable and depended on the particular transgenic plant.

5

The pAGL5Met1as gene could be transformed into other crop species such as *B.napus* and *Zea mays*, leading to an increase in seed size and seed quality in the transgenic plants. In this case it is most preferable to use MET1 and AGL5 orthologous sequences from *B.napus* and *Zea mays*.

10

b) pAP3Met1as

A proportion of transgenic *Arabidopsis* plants containing the pAP3Met1as gene were vegetatively normal and produced flowers with the normal complement of floral organs.

15

Arabidopsis containing pAP3Met1as were pollinated with pollen from wild-type diploid plants or allowed to self pollinate. Endosperm development in the resulting seeds was monitored by confocal microscopy (Scott et al., 1998) and seed weights were measured at maturity. In both cases, endosperms showed a moderate maternal excess phenotype increased peripheral endosperm cell number, precocious cellularisation and chalazal endosperm hypoplasia qualitatively similar to that obtained in 4x-2x crosses between wild type plants (Table 1).

20

The mean weight of mature seed collected from pAP3Met1as plants is less than that of 2x-2x seed.

25

The germination frequency was comparable to that of seed from 2x-2x wild type crosses - about 95-100%.

The pAP3Met1as gene could be transformed into other crop species such as *B.napus* and *Zea mays*, leading to an decrease in seed size in the transgenic plants. In this case it is most preferable to use MET1 and AP3 orthologous sequences from *B.napus* and *Z. mays*.

30

Example 5: Promoting interspecific hybridisation

Tetraploid *Arabidopsis thaliana* were obtained by the method, known to those skilled in the Art, of Colchicine doubling of a diploid plant.

- 5 Cross pollination between tetraploid *Arabidopsis thaliana* (4x *A.thaliana*) and *Arabidopsis lyrata*, results in 100% shrivelled seed (Figure 8A) that fail to germinate (Table 3). Abortion is due to endosperm failure resulting from lethal relative genomic imbalance (Figure 8B). This post-fertilisation hybridisation barrier is overcome by introducing the Met1a/s gene into the 4x
10 *A.thaliana* parent; the resulting plants produce hypomethylated gametes. Cross pollination between a 4x *A.thaliana* Met1a/s seed parent and *Arabidopsis lyrata*, results in plump seed (Figure 8B) which germinate at high frequency (Table 3). This illustrates the utility of hypomethylation, as conditioned by the Met1a/s gene in this example, to promote inter-specific hybridisation between two plants that do not normally form viable hybrids.
15 pAGL5Met1as and pAP3Met1as were transformed into *Brassica campestris* and *Brassica oleracea* via standard methods. Reciprocal crosses between the transgenic individuals of the two species yield plump seeds which germinate to give hybrid plants. Crosses between wild type individuals of the two species result in shrivelled seeds which fail to germinate. Hence the
20 two transgenes overcome the normal barrier to interspecific hybridisation. The same genes could be used in other species or varieties to promote hybridisation.

Table 3 Relaxing genomic imprinting through hypomethylation can promote or prevent hybrid formation

25

Outcome of Cross			
Cross	Endosperm phenotype	Seed viability (% germination)	Hybrids formed?
4x <i>A.thaliana</i> X <i>A.lyrata</i>	ME	0	NO

<i>4xA.thalianaMet1a/s X A.lyrata</i>	Moderate PE	95-100	YES
<i>4xA.thalianaX C.arenosa</i>	Moderate PE	95-100	YES
<i>4xA.thalianaX C.arenosa</i>	Lethal PE	0	NO
<i>4xA.thalianaMet1a/s X C.arenosa</i>	Lethal PE	0	NO

PE, paternal excess as described in Scott et al, 1998. ME, maternal excess as described in Scott et al, 1998.

5

Example 6: Preventing interspecific hybridisation

- 10 Cross pollination between tetraploid *Arabidopsis thaliana* (4x *A.thaliana*) and *Cardaminopsis arenosa*, results in 100% plump seed (Figure 9A) that germinates at high frequency (Table 3). The hybrid is a synthetic version of a naturally occurring hybrid between these two species—*Arabidopsis sueatica* (Chen et al., 1998). Cross pollination between diploid *Arabidopsis thaliana* (2x *A.thaliana*) and *C. arenosa*, results in 100% shrivelled seed that fails to germinate (Table 3). Accordingly, *C. arenosa* can be said to have a genomic strength that is sufficiently high to cause seed abortion when combined with 2x *A. thaliana*, but not when combined with 4x *A. thaliana*. To demonstrate that hypomethylation can prevent cross hybridisation between *A. thaliana* and *C. arenosa* the Met1a/s gene was introduced into 4x *A. thaliana*, and this plant used as seed parent in a cross to *C. arenosa*. Seed from such a cross is 15 100% shrivelled (Fig 9B) and fails to germinate (Table 3). The same gene could be used in other species or varieties to prevent the production of viable hybrid seed.
- 20

Example 7: Maternal hypomethylation promotes autonomous endosperm development

- 25 In the absence of fertilisation, *Arabidopsis* plants heterozygous for the *fie-1* mutation (*fie/FIE*) produce seeds with partial endosperm development (Ohad et al., 1996; 1999; see also Table 3

and Fig. 12 A-C). These 'autonomous' endosperms consist of a severely reduced number of endosperm nuclei (compared to wild type controls) and the endosperm fails to undergo cellularisation. The seed collapses and becomes shrivelled at maturity (Table 4). Consequently, the *fie* mutation conditions only limited endosperm development restricting its utility in the production of autonomous apomictic seed crops or embryoless seed crops. Endosperms produced in plants carrying the *fis1/mea* and *fis2* mutations are very similar to those of *fie/FIE* plants, and hence the utility of these genes is also restricted.

Since "*fie*" endosperms do not contain a paternal genomic contribution one hypothesis is that proper development of the endosperm requires the expression of paternally derived genes that are subject to maternal imprinting.

When plants heterozygous for the *fie* mutation are pollinated with wild type pollen from a 2x wild type plant the ovules carrying the *fie* allele develop into seeds that abort at heart/torpedo stage, while ovules carrying the wild type *FIE* allele develop normally (Ohad et al., 1996; 1999; Table 4 and Fig.10). The aborted seeds express a strong paternal excess phenotype (Table 4; Fig. 10), despite containing only a single paternal contribution. This suggests that a complex situation with respect to imprinting applies within fertilised and unfertilised *fie* endosperms. One hypothesis is that the *fie* mutation lifts imprinting from a proportion of genes normally subject to maternal imprinting: the introduction of a additional paternal genome following fertilisation generates an effective lethal paternal excess such as encountered in a 2x-6x wild type cross (Table 1). The failure of *fie* endosperms to development normally in the absence of fertilisation is also accounted for by this hypothesis, since not all maternally imprinted genes may be derepressed.

Since gametes derived from hypomethylated plants (*Met1as* and *ddm*) appear to have no or highly attenuated imprinting, and therefore act in part as gametes of the opposite sex in endosperms, we hypothesised that such gametes in combination with the *fie* mutation would promote complete endosperm development. In the first experiment, we used pollen from a *Met1as* plant [*FIE/FIE*; *MET1 a/s/MET1 a/s*] to pollinate a *FIE/fie* heterozygote [*fie/FIE*; *MET1*

a/s/MET1 a/s] and found most seeds produced were plump and viable (Table 4; Fig. 11). The seeds segregate 1:1 for the *FIE/FIE:FIE/fie* genotypes, showing that the *fie* allele is transmissible through the seed parent in this cross. The *FIEFIE* seeds display a maternal excess phenotype as expected - endosperm under-development (Table 5) and a reduced seed weight (Table 4), whilst the *Fiefie* seeds display a moderate paternal excess phenotype (Table 5), similar to that observed in a 2xX4x cross between wild type *A. thaliana* plants. When wild type pollen from a diploid plant is used in this cross, the resulting seeds segregate 1:1 for plump/viable:shriveled/inviable and the ovules containing the *fie* mutation produce inviable seed since the plump seeds all contain the wild type *FIE* allele (Table 4; Fig 10). The abortive seeds display a paternal excess phenotype similar to that observed in a 2x-6x cross between wild type *A. thaliana* plants (Figs. 3 and 10; Table 5). Therefore, paternal gametes from Met1as plants appear to rescue *fie* containing seeds from lethality by reducing the magnitude of the paternal excess phenotype. This supports the hypothesis as outlined above.

In the second experiment we combined the *fie* mutation and the Met1as gene into the same individual (see Table 4 and Fig. 12). When these plants were emasculated and left unpollinated 50% of the ovules underwent autonomous endosperm development as expected for ovules carrying the *fie* mutation. Confocal microscopy showed that these seeds contain well developed, cellularised endosperms (Fig. 12), with between 500-700 peripheral nuclei, a cellularisation time of 5-8 days and a volume of chalazal endosperm between 0.01 and 10X that of a seed produced in a 2x-2x cross. The mature seeds were shrivelled, but weighed 15 µg. In contrast, developing ovules of emasculated and unpollinated *Fie/fie* plants contain very under-developed endosperm that do not cellularise (Fig 12). These seeds contain about 200 peripheral endosperm nuclei and no recognizable chalazal endosperm. The mature seeds were shrivelled and weighed 5 µg. The production of an endosperm that has the main features of a wild type endosperm (numerous peripheral endosperm nuclei, cellularisation, and a chalazal endosperm) in plants containing both the *fie* mutation and the Met1as gene shows that the lifting or attenuation of imprinting within the maternal gamete as conditioned by the Met1as gene is sufficient to relieve the developmental block encountered in unpollinated *fie* ovules. This greatly extends the utility of the autonomous endosperm mutants (*fis1*, *fis2*, *fis3*, and *fie*).

Table 4. Enhancement of endosperm development in *fie* mutant seeds by hypomethylation

	Mature seed phenotypes (%) ¹		Seed viability ²		Seed weight µg) ³		Extent of endosperm development (%) ⁴	
	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled seeds	Plump seeds	Shrivelled Seeds	Complete	Partial
FIE/fie X 2x	50	50	95-100	0	25	15	50	50 ⁵
FIE/fie X 2xmet	100	0	95-100	NA	50% = 15 50% = 30	NA	100	0
FIE/fie emasculate	0	100	NA	0	NA	5	0	100 ⁶
FIE/fie: 2xmetHET emasculate	0	100	NA	0	NA	20	100	0

NA, not applicable; FIE/fie, plant heterozygous for the *fie* mutation; 2x, wild type diploid plant; 2xmet, plant homozygous for the Met1as gene; FIE/fie, 2xmetHET FIE/fie heterozygous line containing a single Met1as antisense gene (introduced by crossing FIE/fie and Met1as and recovering appropriate genotype in the F1).

1, scored by eye. 2, determined by germination on soil. 3, measured as described in Scott et al., 1998. 4, determined by confocal microscopy as described in Scott et al, 1998; complete corresponds to normal development as occurs in control crosses, partial refers to abnormal development such as a failure to cellularise or develop chalazal endosperm. 5, resembles lethal paternal excess as occurs in 2x-6x crosses ⁶, as described by Ohad et al, 1999.

Table 5 Endosperm development in crosses involving *fie*, met1a/s and wild type plants.

	<i>fie/FIE X FIE/FIEmet/met</i>	<i>fie/FIE X FIE/FIE</i>		
	<i>FIE/FIE</i> seeds	<i>fie/FIE</i> seeds	<i>FIE/FIE</i> seeds	<i>fie/FIE</i> seeds
Maximum number of P.E nuclei	192	637	447	408
Timing of endosperm cellularisation	3-4 DAP	7-8 DAP	5-6 DAP	> 10 DAP
Size of chalazal Endosperm ¹	0.05-0.1X	3-4X	1X	10-15X

¹, area of maximum cross-section relative to wild type

5 **Example 8: Production of plants that combine the *fie* mutation and the female germ-line specific demethylating gene, AGL5Met1a**

10 Plants heterozygous for the *fie* mutation and hemizygous for the pAGL5Met1as gene were generated by making crosses between *FIE/fie* plants as pollen parent and plants homozygous for the pAGL5Met1as gene as seed parent. These plants were vegetatively normal and produced normal flowers. When emasculated 50% of the ovules initiated seed development without fertilisation. Confocal microscopy showed that endosperm development was extensive, resulting in a large (500-700 nuclei) cellularised endosperm.

15 The pAGL5Met1as gene could be introduced into crop species, such as *B.napus* and *Zea mays* in which expression of the *FIE* gene, or any of the genes that condition autonomous endosperm development, is suppressed or absent through mutation or the use of transgenic technologies, to produce promote apomixis or embryoless (pseudoapomictic) seed. Preferably the pAGL5Met1as construct contains *B.napus* or *Z.mays* MET1 and AGL5 orthologous sequences

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Example 9 The use of FIE down-regulation to paternalise female gametes (polar nuclei) - to increase endosperm size and seed weight.

When plants heterozygous for the *fie* mutation (Ohad et al., 1996; 1999) are pollinated with pollen from a 2x wild type plant the ovules carrying the *fie* allele develop into seeds that abort at heart/torpedo stage, while ovules carrying the wild type *FIE* allele develop normally (Ohad et al, 1996; 1999; Table 4 and Fig. 10). The aborted seeds express a strong paternal excess phenotype (Table 4; Fig. 10), despite containing only a single paternal contribution. This suggests that a complex situation with respect to imprinting applies within fertilised and unfertilised *fie* endosperms. This is explained by proposing that the *fie* mutation lifts imprinting from genes normally subject to maternal imprinting (the maternal gametes are thus strongly paternalised): the introduction of a additional paternal genome following fertilisation generates an effective lethal paternal excess (2maternal;3paternal) such as encountered in a 2x-6x wild type cross (2m:3p) (Table 1).

Since gametes derived from hypomethylated plants (Met1as and ddm) appear to have no or highly attenuated imprinting, and therefore act in part as gametes of the opposite sex in endosperms, such gametes in combination with the *fie* mutation could promote complete endosperm development. In the first experiment, pollen from a Met1as plant [*FIE/FIE*; *METI* a/s/*METI* a/s] is used to pollinate a *FIE/fie* heterozygote [*fie/FIE*; *METI* a/s/*METI* a/s] and most seeds produced were plump and viable (Table 4; Fig. 11). The seeds segregate 1:1 for the *FIE/FIE*:*FIE/fie* genotypes, showing that the *fie* allele is transmissible through the seed parent in this cross. The *FIEFIE* seeds display a maternal excess phenotype as expected - endosperm under-development (Table 5) and a reduced seed weight (Table 4), whilst the Fiefie seeds display a moderate paternal excess phenotype (Table 5), similar to that observed in a 2xX4x cross between wild type *A. thaliana* plants. When wild type pollen from a diploid plant is used in this cross, the resulting seed segregate 1:1 for plump/viable:shriveled/inviabale and the ovules containing the *fie* mutation produce inviable seed since the plump seeds all contain the wild type *FIE* allele (Table 4; Fig 10). The abortive seeds display a paternal excess phenotype similar to that observed in a 2x-6x cross between wild type *A. thaliana* plants (Figs. 3 and 10; Table 5). Therefore, paternal gametes from Met1as plants appear to rescue *fie* containing seeds from lethality by reducing the magnitude of the paternal excess phenotype. As the *fie* mutation appears to cause strong paternalisation of the maternal gametes (polar nuclei), wild-type *FIE* may participate directly in maternal imprinting (as part of the imprinting complex).

The paternalisation of the polar nuclei by the *fie* mutation is more extensive than that achieved by met^{Ia}/s since a *fie*X2x cross results in lethal paternal excess (Table 4; Fig 10), but a met^{Ia}/s X2x cross produces viable paternal excess, with increased endosperm size and seed weight (Table 1). Thus the degree of paternalisation of the polar nuclei determines the outcome of crosses with pollen from diploid wild type plants: moderate paternalisation (e.g Met^{Ia}/s) produces a large viable seed due to moderate paternal excess in the endosperm, whereas strong paternalisation (e.g *fie* null mutation) results in seed lethality due to excessive paternal excess in the endosperm. Modulating FIE expression may have application in manipulating endosperm size and seed weight. The *fie* mutation used is a null allele (*fie*-1; Ohad *et al.*, 1999) - no functional FIE protein is produced, resulting in strong paternalisation of the polar nuclei, and seed lethality in crosses with wild type pollen from a diploid plant. Reducing, but not eliminating the expression of *FIE* results in moderate paternalisation of the polar nuclei; the exact level of paternalisation being directly related to the amount of FIE protein expression during female gametogenesis. Reduction in FIE expression can be achieved using a number of well known methods such as antisense RNA expression against the sense *FIE* RNA transcript. Incremental reduction in FIE expression, by making use of for example different, more or less effective, anti-sense lines, identifies a level of FIE expression that is optimal for producing viable seeds with a maximally increased endosperm size and seed weight.

Suitable anti-sense genes would comprise the FIE promoter driving transcription of the anti-sense *FIE* transcribed region. Other genes suitable to reduce the levels of *FIE* expression and deliver levels of paternalisation of polar nuclei intermediate between a *FIE* null allele and the wild type *FIE* allele include genes encoding fragments of the FIE protein which recognise and bind to imprinted genes, but are ineffective in promoting their non-expression in the endosperm (e.g. because the repressive complex cannot form or cannot be maintained).

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